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The herbal compound geniposide rescues formaldehyde-induced apoptosis in N2a neuroblastoma cells

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The herbal medicine Tong Luo Jiu Nao (TLJN) contains geniposide (GP) and ginsenoside Rg1 at a molar ratio of 10:1. Rg1 is the major component of another herbal medicine, panax notoginseng saponin (PNS). TLJN has been shown to strengthen brain function in humans, and in animals it improves learning and memory. We have previously shown that TLJN reduces amyloidogenic processing in Alzheimer's disease (AD) mouse models. Together this suggests TLJN may be a potential treatment for patients with dementia. Because chronic damage of the central nervous system by formaldehyde (FA) has been presented as a risk factor for age-associated cognitive dysfunction, in the present study we investigated the protective effect of both TLJN and GP in neuron-like cells exposed to FA. FA-exposed murine N2a neuroblastoma cells were incubated with TLJN, its main ingredient GP, as well as PNS, to measure cell viability and morphology, the rate of apoptosis and expression of genes encoding Akt, FOXO3, Bcl2 and p53. The CCK-8 assay, cytoskeletal staining and flow cytometry were used to test cell viability, morphology and apoptosis, respectively. Fluorescent quantitative real-time PCR (qRT-PCR) was used to monitor changes in gene expression, and HPLC to determine the rate of FA clearance. Treatment of N2a cells with 0.09 mmol L^{-1} FA for 24 h significantly reduced cell viability, changed cell morphology and promoted apoptosis. Both TLJN and GP conferred neuroprotection to FA-treated N2a cells, whereas PNS, which had to be used at lower concentrations because of its toxicity, did not. Our data demonstrate that TLJN can rescue neuronal damage caused by FA and that its main ingredient, GP, has a major role in this efficacy. This presents purified GP as a drug or lead compound for the treatment of AD.

Alzheimer's disease, apoptosis, formaldehyde, geniposide, neuroprotection, panax notoginseng saponin, Tong Luo Jiu Nao

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Formaldehyde (FA) is an essential metabolic intermediate generated endogenously from serine, glycine, methionine and choline. It is also a metabolic by-product of xenobiotic chemicals and proteins in the process of demethylation [1]. FA is a colorless, irritating gas with a high oxidation capacity. The specific gravity of FA is 1.06, the boiling point is –21°C and the melting point is –91°C. FA is volatile and easily soluble in water. The saturated 35%–40% aqueous solution of FA is known as formalin [2]. The annual average concentration of FA in urban air is about 0.005–0.01 mg m^{-3}, and generally does not exceed 0.03 mg m^{-3} [3].

As a high-volume industrial chemical, FA can enter the body in a number of different ways depending on the type

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of environmental exposure. In humans, inhalation of FA has been reported to result in changes in attention, memory, balance, mood and sleep [4]. For example, those who are chronically exposed to formalin display symptoms of amnesia as well as dysfunction in other cognitive processes [5], and often present with dizziness and impaired balance [6]. Studies carried out by Malek and colleagues demonstrated that exposure to FA can cause a decline in memory function in rats, which is accompanied by a loss of hippocampal and cortical neurons [7]. Furthermore, in mice, inhalation of FA has been shown to affect the morphology of olfactory and hippocampal neurons, decrease the expression of CaMKII in the hippocampal CA3 region, and impair performance in behavioural learning and memory tasks such as the Morris water maze and six-arm radial maze [8]. Khokhlov and colleagues have shown that FA levels are significantly increased in the blood and cerebrospinal fluid of multiple sclerosis patients with memory decline [9].

Although the mechanisms underlying the neurotoxic effect of FA are not fully understood, previous studies have shown that FA can cross-link proteins, DNA and unsaturated fatty acids. Because of its high reactivity, FA will rapidly interact with cells lining the epithelial surface of the nose and lung when inhaled [10,11]. Interestingly, low doses of FA have been shown to induce the aggregation of the microtubule-associated protein tau, which is implicated in Alzheimer's disease (AD), and to lead to cytotoxic oligomers in human SH-SY5Y neuroblastoma cells [12,13].

As previously described by Tong and colleagues, in humans uric FA increases with age (>65 years old), and levels of FA increase in mice as they become older (8 months old) [14]. Similarly, rat hippocampal FA has also been shown to increase with age (>12 months old). Thus, chronic impairment of the central nervous system induced by endogenous FA has been suggested to be a risk factor for age-related cognitive impairment [15,16].

Chinese herbal medicine is part of a larger healing system called traditional Chinese medicine (TCM). Tong Luo Jiu Nao (TLJN) is a herbal compound that is prepared under the guidance of "the pathogenesis theory of heat-toxic affecting the brain collaterals" (impairment of brain and its function). It contains a variety of ingredients, with the main active ingredients being geniposide (GP) and geniposide Rg1 (Rg1) at a molar ratio of 10:1 [17]. Rg1 is the major component of the herbal medicine panax notoginseng saponin (PNS), which is reported to be neuroprotective [18] and is used in the treatment of cardiovascular diseases in China [19].

TLJN is made by mixing extracts of gardenia and pseudoginseng (see Materials and methods for details). The biological effects of the extracts complement each other: pseudoginseng "promotes blood circulation, reinforces insufficiency and strengthens the brain", and gardenia "relieves inflammatory symptoms and eliminates toxicity" [12,20]. The two extracts can relieve both the primary and secondary symptoms of inflammation, as well as provide temporary relief and clear the "meridian" (a TCM term used to describe the energy flow around the body; see Supporting Information), conforming with the Chinese therapeutic principles of "to pass to fill" (the more circulation is unobstructed, the better nutrients can be supplied). The extracts also "generate new blood, strengthen the brain, improve intelligence, strengthen the spleen and kidney, nourish the heart, and calm the nerves" [18,19].

Although how TLJN, GP and PNS work at a molecular level is unknown, the Akt signaling pathway has been recognized as a critical regulator of cell survival. Akt, also known as protein kinase B (PKB), is a serine/threonine-specific kinase. The Akt family is composed of at least three members: Akt1/PKBα, Akt2/PKBβ and Akt3/PKBγ, with the latter being enriched in the brain and testis [21]. Akt has a key role in signal transduction and tumor formation through either the stimulation of cell regeneration or the inhibition of apoptosis [22].

There is collective evidence that endogenous FA levels increase with age, and that this increase is associated with memory decline [14]. Using the FA stress model of mouse N2a neuroblastoma cells, we report that GP and TLJN, but not PNS, have a protective effect on N2a cells exposed to FA. This data suggest that GP may be the active ingredient in TLJN, which has previously shown efficacy in delaying or ameliorating age-related cognitive impairment.

1 Materials and methods

1.1 Materials

GP and PNS were obtained from the National Institutes for Food and Drug Control (Beijing, China). Gardenia and pseudoginseng extracts were purchased from Nanjing Zelang Medical Technology Limited Company (China). GP and PNS were obtained from the National Institutes for Food and Drug Control (China). TLJN was prepared by mixing extracts of gardenia and pseudoginseng in proportions determined using HPLC in previous experiments [23]. TLJN contains 10 parts GP to one part Rg1. The active ingredient of PNS is Rg1.

TLJN is able to activate Aβ-degrading enzymes in rat AD models [17]. As a culture medium for N2a cells, Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), EDTA-pancreatin and Penicillin-Streptomycin (PS) were used (Gibco Co., USA). The Cell Counting Kit-8 (CCK-8) for measuring cell viability was obtained from Dojindo Co. Annexin V-FITC/PI Apoptosis Assay Kit was purchased from Zoman Bio. Hoechst 33258 was obtained from Sigma and Alexa Fluor® 488 Phalloidin from Invitrogen. Trizol reagent for RNA extraction was purchased from Invitrogen. Other reagents for RT-qPCR including RNase-free Water, EasyScript First-Strand cDNA Synthesis Super-

Mix, 2× Easy Taq PCR SuperMix and TransStartTM Green qPCRSuperMix UDG were purchased from Transgen.

1.2 Cell culture

N2a cells were grown in DMEM containing 10% (v/v) FBS and PS, maintaining 5% CO_2/90% humid air at 37°C. Cells were plated into 96-well plates at a density of 1.0×10^4 cells per well for cell viability assay, or into 60 mm culture dishes at a density of 1×10^6 cells/well for the cell apoptosis assay, RT-qPCR and HPLC, or in confocal culture dishes at a density of 3×10^5 cells per dish to determine cell morphology. After 24 h, the culture medium was replaced with serum-free DMEM supplemented with different concentrations of FA and compounds as indicated.

1.3 Cell viability assay

The viability of cells was evaluated using the CCK-8 kit. In brief, 10 μL of the CCK-8 solution was added to each well of 96-well plates, and incubated for 4 h at 37°C. According to the manufacturer's protocol, simultaneous absorbance readings were measured at 450 and 630 nm using a microplate reader. Median lethal concentration (LD50) of FA on N2a cells, the concentration of drug safety use, and the effective concentration of the drugs rescuing the cells in the presence of FA were determined by analyzing different doses of the drugs used.

1.4 Apoptosis assay

N2a cells (plated at a density of 1×10^6) were grown in 60 mm plates overnight. The culture medium was then replaced with serum-free DMEM supplemented with 0.09 mmol L^{-1} FA and different effective concentrations of drugs (200 μmol L^{-1} TLJN, 200 μmol L^{-1} GP or 20 μmol L^{-1} PNS) for 24 h. The analysis of apoptosis was carried out by flow cytometry using the Annexin V-FITC/PI apoptosis detection kit. Cells were harvested, counted, and resuspended in 1× binding buffer at a concentration of 1×10^6 cells mL^{-1}. Then, 5 μL of Annexin V-FITC was added to the cell suspension (500 μL), which was gently mixed and incubated for 10 min at room temperature in the dark. Finally, propidium iodide (PI) was added (10 μL) and samples were analyzed by flow cytometry (FACSCalibur, BD Biosciences, USA) within 1 h.

1.5 Analysis of cell morphology and numbers

After culturing N2a cells in DMEM complete medium for 24 h, the cells were transferred into serum-free DMEM supplemented with 0.09 mmol L^{-1} FA and different effective concentrations of the respective drugs (200 μmol L^{-1} TLJN, 200 μmol L^{-1} GP, and 20 μmol L^{-1} PNS, respectively). After 24 h, the cells were fixed with cold 4% (w/v) paraformaldehyde (PFA) for 15 min at 4°C. F-actin was visualized with FITC-phalloidin (Alexa Fluor® 488 Phalloidin, USA) and nuclei with Hoechst 33258. All images were obtained using laser scanning confocal microscopy (LSCM; Olympus FV1000). Three to four fields (10× objective lens) were randomly selected from each dish for imaging. Counting of nuclei was carried out using the graphic software IPP6.0 to compare cell adhesion in the experimental and control groups.

1.6 RT-qPCR analysis

Expression levels of the genes encoding Akt, FOXO3, Bcl2 and p53 were detected by qRT-PCR. Total RNA was extracted from N2a cells using Trizol reagent. The integrity of RNA was examined by identifying intact 18S and 28S ribosomal RNA bands using agarose gel electrophoresis. Purity and quantity were estimated by measuring the absorbance at 260/280 nm. RNA was reverse-transcribed into cDNA using M-MLV reverse transcriptase. PCR was performed using Taq DNA polymerase. The PCR primers were designed with Primerpremier 5.0 software and primer bank (http://pga.mgh.harvard.edu/primerbank/), and were synthesized by Shanghai Sangon Inc. The primers for *bcl-2* were as follows: forward: 5′-TGTGTGGAGAGCGTCAACAG-3′ and reverse: 5′-CAGACATGCACCTACCCAGC-3′; for 18S RNA, forward: 5′-CAGCCACCCGAGATTGAGCA-3′ and reverse: 5′-TAGTAGCGACGGGCGGTGTG-3′. Fluorescence quantitative PCR was carried out using the TransStartTM Green qPCRSuperMix UDG kit (Transgen Inc., China) on the real-time fluorescent quantitative PCR instrument Corbett6600 (Corbett Research, Germany).

1.7 Analysis of formaldehyde (FA) concentration

The concentration of FA in the medium was analyzed by HPLC using Carbon-18 columns (Agilent). The sample buffer consisted of acetonitrile, 2,4-dinitrophenylhydrazine (1.0 g L^{-1}) and trichloroacetic acid (10%). Media were harvested and mixed with sample buffer to analyze FA by HPLC.

1.8 Statistical analysis

Analysis of variance was carried out using SPSS 16.0. The data is expressed as the mean±standard deviation (SD) and one-way analysis of variance was used for comparison between groups. Values of $P<0.05$ were considered statistically significant.

2 Results

2.1 Toxicity of FA and the three herbal compounds

We first determined the toxicity of FA in a cellular system

and then assayed the effects of the three herbal compounds GP, PNS, and TLJN on cell viability using the CCK-8 assay kit. N2a cells were cultured in media containing increasing concentrations of FA for 24 h. We defined the cell density in the absence of FA as 100%. By titrating FA, we found that the median lethal concentration (LD50) was 0.086 mmol L^{-1} (Figure 1). For practical purposes we used 0.09 mmol L^{-1} FA, which is equivalent to a cell survival ratio of 46%, for all follow-up experiments (unless stated otherwise).

We next performed a toxicity assay for GP, PNS and TLJN (Figure S1 in Supporting Information). N2a cells were exposed to increasing concentrations of GP, and cell viability was determined after 24 h of exposure (Figure 2). GP at concentrations up to 200 μmol L^{-1} did not affect cell viability at all (Figure 2A); however, even at concentrations as low as 15 μmol L^{-1}, treatment with PNS resulted in decreased cell viability (P<0.01) (Figure 2B). TLJN treatment did not have any toxic effects on N2a cells at concentrations of up to 200 μmol L^{-1} (Figure 2C). Therefore, concentrations of GP and TLJN of up to 200 μmol L^{-1} were considered to be non-toxic to these cells and also to mitochondria, as the CCK-8 kit primarily measures mitochondrial activity.

Next, we asked whether the drugs would decrease levels of FA produced by the N2a cells themselves. We detected endogenous FA in the medium by HPLC-UV as described previously [24]. All three drugs significantly decreased FA concentrations in the N2a cell culture medium compared with the untreated group (P<0.01) (Figure 3A).

To identify whether the drugs directly reacted with FA, each drug was incubated with FA in the absence of cells for 24 h and then the residual FA was determined. As shown (Figure 3B), none of the drug treatments resulted in a decrease in FA, suggesting that the decrease in FA in the medium is a cellular response.

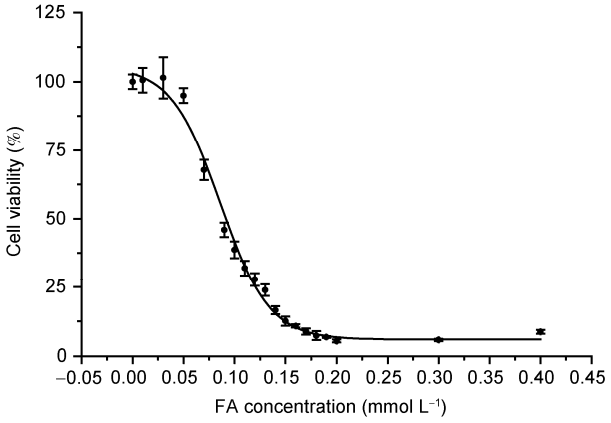

Figure 1 Median lethal concentration (LD50) of formaldehyde *in vitro*. N2a cells were plated onto 96-well plates at a density of 1.0×10^4 cells per well for cell viability assay. After 24 h, the culture medium was replaced with serum-free DMEM supplemented with increasing concentrations of formaldehyde (FA). Cell viability was determined with the CCK-8 assay after 24 h of incubation. The LD50 of FA *in vitro* was determined as 0.086 mmol L^{-1}. Data are expressed as mean±SD from at least three independent experiments.

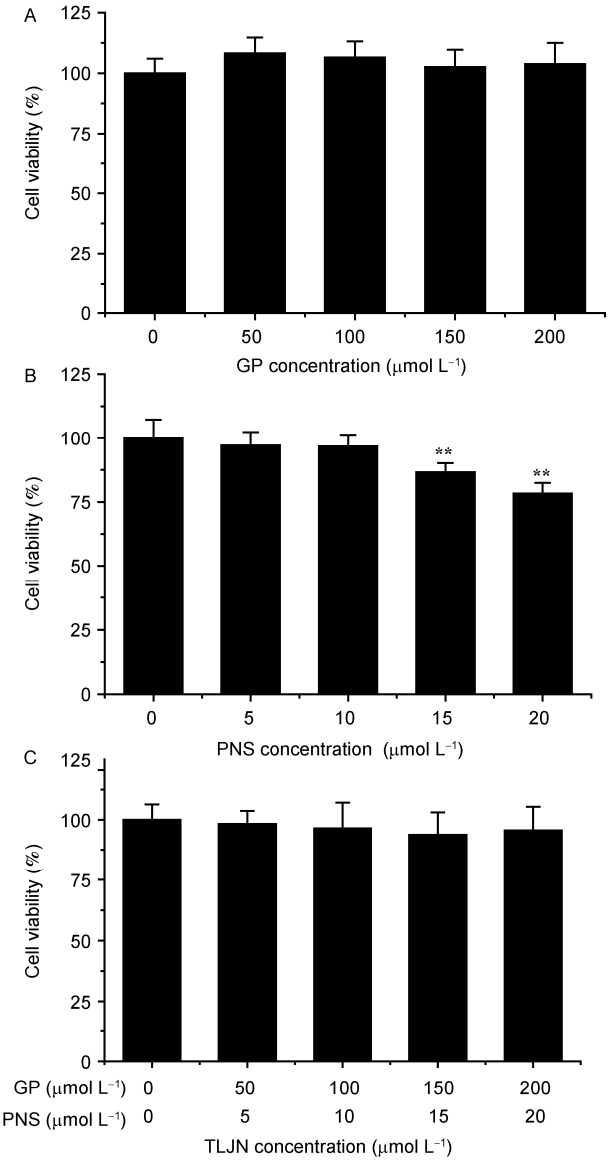

Figure 2 Determining drug safety. Conditions for cell culture were the same as those in Figure 1, except that the cells were treated with a range of concentrations of three drugs geniposide (GP), panax notoginseng saponin (PNS) and Tong Luo Jiu Nao (TLJN), a herbal medicine containing GP and Rg1 at a molar ratio of 10:1 (Figure S1 in Supporting Information). Changes in N2a cell viability following treatment with different concentrations of GP (A), PNS (B) and TLJN (C). Cells without drug treatment were used as control. Data are expressed as mean±SD from at least three independent experiments. **, P<0.01 vs. the control group.

2.2 GP increases viability of FA-treated N2a neuroblastoma cells

Next, we investigated whether GP has a neuroprotective effect on cells to which FA was added exogenously. For that, N2a cells were plated at a density of 1×10^4/well and grown in serum-free DMEM medium in the presence of

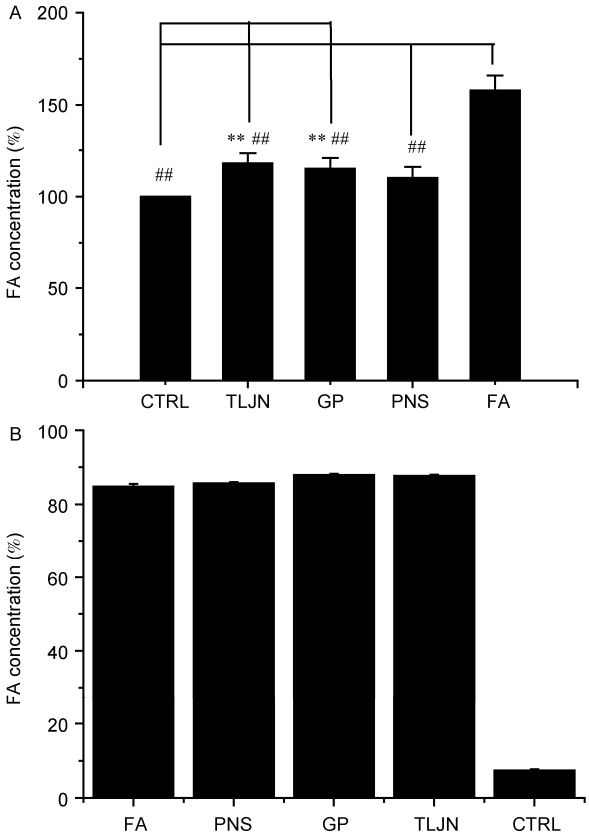

Figure 3 Tong Luo Jiu Nao (TLJN) decreases the concentration of formaldehyde in the medium. N2a cells were cultured for 24 h and incubated with geniposide (GP), panax notoginseng saponin (PNS) or Tong Luo Jiu Nao (TLJN) in the presence of exogenous formaldehyde (FA). The concentration of FA in the medium was determined by HPLC. Cells cultured in the absence of exogenous FA were used as control (Ctrl) (A). Using the same conditions, each of the compounds TLJN, GP and PNS were directly incubated with FA in a cell-free system for 24 h and then aliquots were taken for measurements of concentrations of FA (B). Data are expressed as mean±SD from three independent experiments. **, P<0.01 vs. the control; ##, P<0.01 vs. the group treated with FA alone.

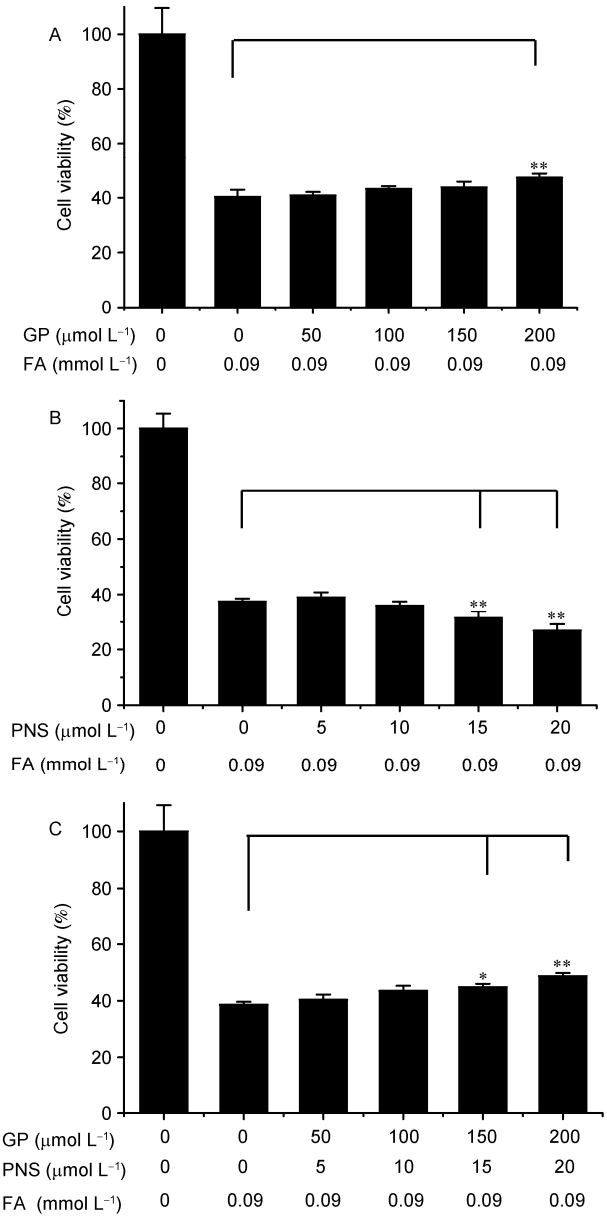

Figure 4 Geniposide rescues the viability of N2a cells treated with formaldehyde. N2a cells were incubated with increasing concentrations of GP (A), PNS (B), or TLJN (C) with 0.09 mmol L^{-1} formaldehyde (FA) overnight. The viability of cells was evaluated using the CCK8 assay. Cells without the treatment were used as control. Data are expressed as mean±SD from three independent experiments. *, P<0.05; **, P<0.01 vs. the control.

0.09 mmol L^{-1} FA supplemented with GP. Changes in cell viability were monitored using the CCK-8 viability kit. Treatment with increasing concentrations of GP showed that at 200 μmol L^{-1}, cell viability was significantly increased (6%, P<0.01) compared to FA-treated cells (Figure 4A). In contrast, cell viability was significantly decreased after 24 h incubation in either 15 μmol L^{-1} or 20 μmol L^{-1} PNS (8% or 14% respectively, P<0.01) (Figure 4B). This again demonstrates that PNS alone has a toxic effect on the cells. TLJN at concentrations of 150 μmol L^{-1} (7%, P<0.05) and 200 μmol L^{-1} (12%, P<0.01) increased cell viability remarkably compared with the FA-only control (Figure 4C), suggesting an ability to rescue N2a cells from FA-induced injury.

2.3 TLJN and GP reduce apoptosis in FA-treated N2a cells

To determine whether a reduction in apoptosis contributes to the protective effect of TLJN, we measured apoptosis in FA-treated N2a cells by flow cytometry. The data indicate that 18.28% of non-treated N2a cells were apoptotic when serum-free DMEM was used (Figure 5A). The percentage of apoptosis increased to 29.23% for FA-treated cells (Figure 5B); however, incubation with 200 μmol L^{-1} TLJN suppressed apoptosis, with the percentage of apoptotic cells decreasing to 12.77% (Figure 5C). Furthermore, incubation with 200 μmol L^{-1} GP in the presence of FA decreased the percentage of apoptotic cells to 13.92% (Figure 5D). Treat-

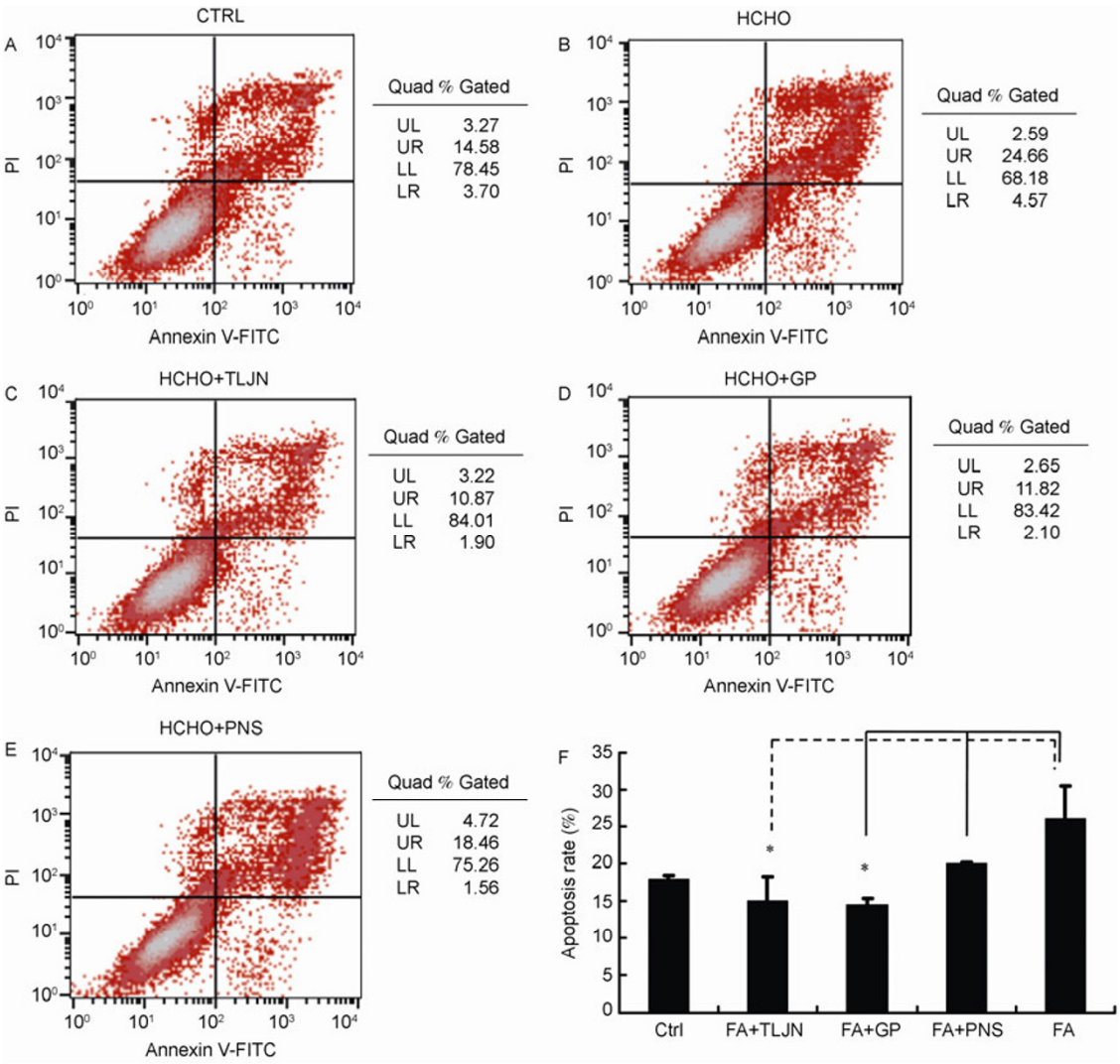

Figure 5 TLJN inhibits apoptosis of N2a cells in the presence of formaldehyde. N2a cells were plated at 1×10^6 per in 60 mm plate and grown overnight. The culture medium was then replaced with serum-free DMEM supplemented with 0.09 mmol L^{-1} formaldehyde (FA) and drugs at different effective concentrations for 24 h. Apoptosis was determined by flow cytometry using the Annexin V-FITC/PI apoptosis detection kit where the rate of apoptosis is calculated by the fraction of UL+UR/total cells. Cells with no treatment were used as control (A). Cells were incubated with FA alone (B) or together with 200 μmol L^{-1} TLJN (C), 200 μmol L^{-1} GP (D), or 20 μmol L^{-1} PNS (E). Data are expressed as mean±SD from three independent experiments (F).

ment with 20 μmol L^{-1} PNS, surprisingly, caused a slight decrease in the percentage of apoptosis (20.02%) compared to the control group (Figure 5E). These findings are in agreement with those obtained with the CCK-8 viability assay. The data suggest that GP can reduce apoptosis in FA-treated N2a cells (Figure 5F).

2.4 Morphology and cell numbers are restored following treatment with TLJN and GP

After incubating N2a cells with TLJN, GP or PNS for 24 h, the cells were stained with phalloidin and Hoechst 33258 and analyzed using laser scanning confocal microscopy (Figure 6). Nuclei appeared shrunken in cells treated with FA (Figure 6A), whereas round nuclei were observed in the saline-treated control group (Figure 6C). When FA-treated N2a cells were co-incubated with GP or TLJN, they had significantly fewer malformed nuclei (Figure 6B and D). Treatment with PNS did not ameliorate the toxic effect of FA (Figure 6E). Similar to what has been shown previously [25], FA-treated cells grew many short processes and the cell density was significantly decreased ($P<0.01$, $n=3$) (Figure S2 in Supporting Information). In general, GP and TLJN did not seem to ameliorate the changes in the F-actin morphology induced by FA.

After FA treatment, cell number was significantly reduced (Figure 6F). TLJN treatment resulted in an increase in cell number when compared with cells treated only with FA ($P<0.01$), indicating that TLJN exerted a protective effect on FA-injured N2a cells. A similar effect was also found in cells supplemented with GP ($P<0.01$); however, there was no significant difference between FA-treated and

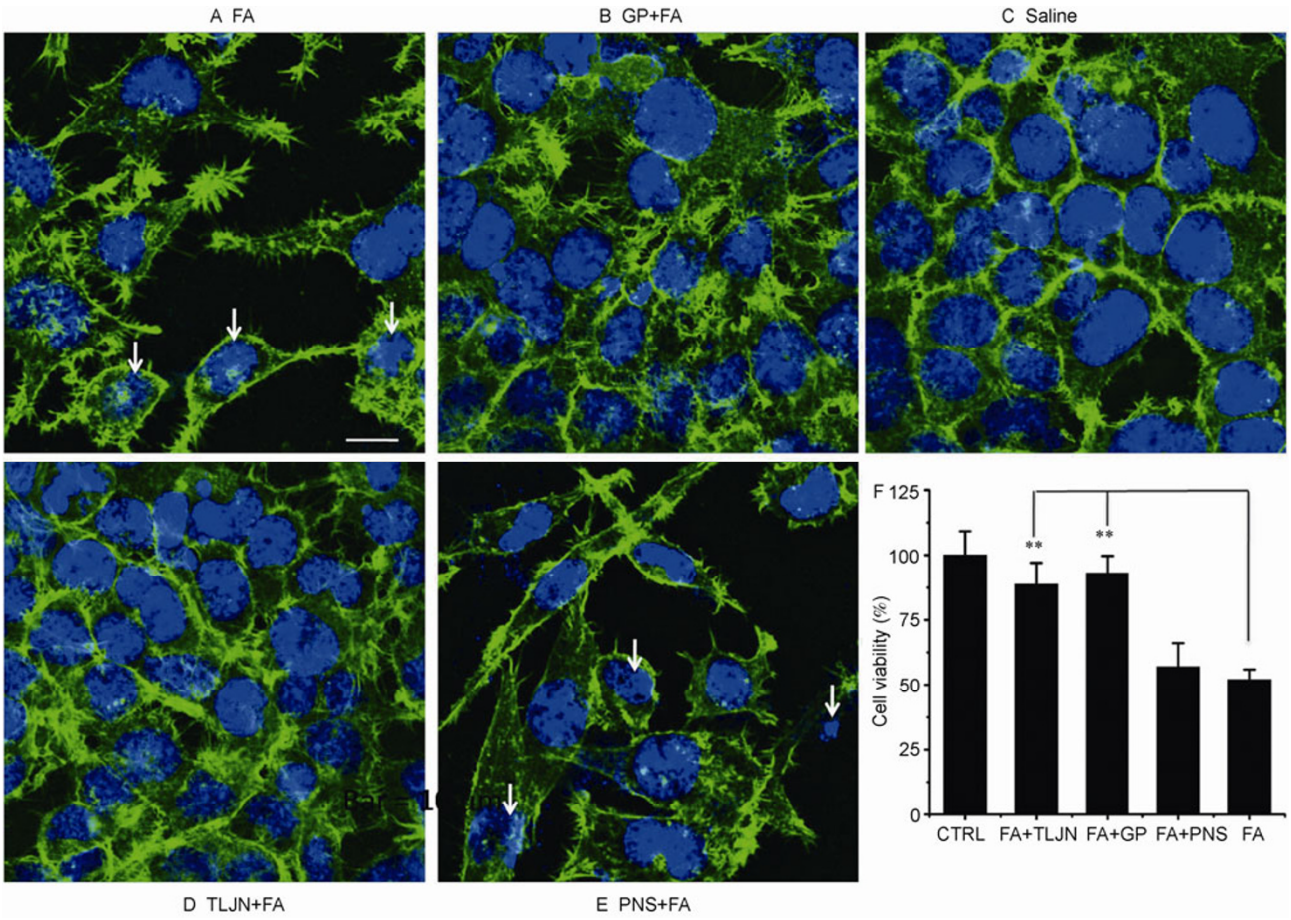

Figure 6 TLJN rescues the morphology and increases the number of formaldehyde-treated N2a cells. Cells were plated onto confocal culture dishes at a density of 3×10^5 cells per dish to determine cell morphology. Conditions for cells treatments were the same as for Figure 4, except that the cell morphology analysis was carried out by staining F-actin and nuclei. Cells were incubated with GP+FA, TLJN+FA, PNS+FA, FA alone and saline, as indicated. Numbers of surviving cells were also recorded (F). Cells with no treatment were used as control. Data are expressed as mean±SD from three independent experiments. **, $P<0.01$ vs. the control.

FA plus PNS-treated cells. This indicates that GP is the key component of TLJN that protects cells from the adverse effects of FA, which is consistent with data from our CCK-8 and flow cytometry assays.

2.5 Expression of Akt, FOXO3, Bcl2 and p53 in the presence of GP

The Akt signaling pathway has a critical role in cell survival. Akt, FOXO3, Bcl2 and p53 are apoptosis regulator proteins in this pathway; therefore, we determined mRNA expression of these genes using rRT-PCR. Total RNA was extracted from N2a cells using the Trizol reagent. The integrity of RNA was examined by identifying intact 18S and 28S ribosomal RNA bands by gel electrophoresis (Figure S3 in Supporting Information). We found that expression levels of Akt mRNA in cells treated with FA were significantly decreased compared with the control ($P<0.01$) (Figure 7, Table 3; Figure S4 in Supporting Information). Treatment with TLJN and GP, on the other hand, restored Akt mRNA expression of FA-injured cells to normal levels

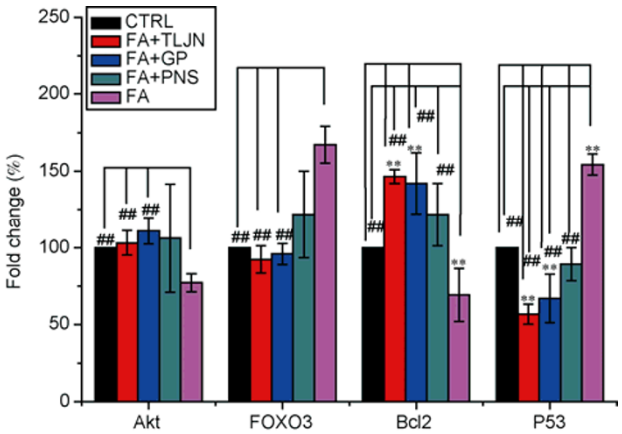

Figure 7 TLJN regulates Akt, FOXO3, Bcl2 and p53 expression. Conditions for the treatment of cells were as described in Figure 1. Total RNA was extracted from N2a cells using Trizol reagent. The integrity of RNA was examined by identifying intact 18S and 28S ribosomal RNA bands by gel electrophoresis (Figure S1 in Supporting Information). The expression levels of the genes encoding Akt, FOXO3, Bcl2 and p53 were detected by qRT-PCR. Cells with no treatment were used as control. Data are expressed as mean±SD from three independent experiments. **, $P<0.01$ vs. the control; ##, $P<0.01$ vs. the group treated with FA alone.

(P<0.01). Treatment with PNS did not have a significant effect when compared to the control group. FA elevated mRNA levels of FOXO3 significantly (P<0.01) (Table 4; Figure S5 in Supporting Information). Treatment with TLJN or GP restored FOXO3 mRNA expression of FA-injured cells to normal levels (P<0.01). Compared to saline-treated controls, Bcl2 mRNA levels were down-regulated by FA (Table 5; Figure S6 in Supporting Information), but increased following treatment with GP or TLJN (P<0.01). FA induced a robust increase in p53 mRNA levels compared to the control, whereas levels in the TLJN- or GP-treated groups were much lower (P<0.01) (Table 6; Figure S7 in Supporting Information).

Together the data suggest that, compared with the control, exposure to FA down-regulates the expression of Akt and Bcl2, and up-regulates FOXO3 and p53 expression, thus contributing to cell apoptosis. We hypothesize that both GP and TLJN significantly protect N2a cells from the neurotoxic effects of FA by inducing anti-apoptotic processes.

3 Discussion

As humans age, the regulation of endogenous FA gradually declines. This age-related impairment may be, in part, due to damage caused by oxidative stress [28]. We recently published data suggesting that regularly drinking water improves clearance of FA from the kidney, potentially delaying age-related cognitive impairment [29]. Human autopsies have shown significantly increased concentrations of FA in the hippocampus of patients with dementia compared to controls [30]. In the present study, we report that GP decreases the concentration of FA in cultured N2a cells, suggesting that this herbal compound may scavenge endogenous FA and have the potential as a treatment for age-related dementia by decreasing brain FA.

As mentioned above, in contrast to GP and TLJN, we did not find PNS to protect N2a cells from FA-induced stress because PNS itself was toxic at concentrations as low as 20 μmol L^{-1}. Higher concentrations could not be tested, making a comparison with GP difficult. The CCK-8 assay primarily measures mitochondrial activity, a function that is improved by GP and TLJN, and we therefore suggest that the mechanism by which FA exerts its toxicity lies upstream of mitochondrial dysfunction.

Akt is an important kinase that regulates survival and differentiation. Our experiments demonstrate a close relationship between Akt levels and cell apoptosis induced by FA. We found that by treating N2a cells with FA for 24 h, levels of Akt decreased and expression of its downstream gene FOXO3 increased, leading to lower expression of anti-apoptotic genes, and up-regulation of the pro-apoptotic gene *p53*. When FA-treated N2a cells were co-incubated with FA and either GP or TLJN, Akt was up-regulated, FOXO3 down-regulated, expression of the anti-apoptotic gene Bcl2 was increased, and expression of the pro-apoptotic gene p53 decreased. In contrast, PNS had no anti-apoptotic effect. Our results reveal that TLJN and GP block FA-induced apoptosis, possibly via the Akt signaling pathway. In follow-up experiments, the efficacy of Akt inhibitors could be tested to consolidate the role of this pathway in conferring resistance to apoptosis. In addition, our data support the concept of the "endogenous formaldehyde metabolic disorders theory" underlying the pathogenesis of sporadic AD [16]. We propose that studying the Akt signaling pathway is crucial in monitoring the efficacy of any treatment of AD.

Inhibition of the Akt signaling pathway has been reported in patients with AD. More specifically, levels of Akt and phospho-Akt, as well as Akt activity, were all reported to be decreased relative to controls [31]. Furthermore, the Akt signaling pathway has been shown to modulate the hyperphosphorylation of tau induced by nanomolar concentrations of naturally secreted amyloid-β [32], although hyperphosphorylation of tau can be promoted by many additional factors [33]. We found that GP up-regulates Akt and Bcl2, and down-regulates FOXO3 and P53. Thus, GP protects N2a cells at multiple steps. On the one hand, up-regulation of Akt leads to an increase of Bcl2 expression [34], which promotes cell survival [35] but on the other hand, down-regulation of FOXO3 [36] and p53 [37,38] results in the promotion of apoptosis. This shows that GP has multiple effects on cell death and survival.

As mentioned above, PNS seems to show an effect similar to TLJN and GP on the Akt pathway, but the PNS data were not as consistent and the standard deviation was larger, making the difference non-significant (Figure 7). PNS did not show a significant rescue of the FA-treated cells compared to PG and TLJN. So, although it appears that PNS is not involved in activation of the Akt pathway, further investigation should be undertaken.

Our previous studies have indicated that in an AD rat model, TLJN injection can significantly improve learning and memory in the Y-maze [17]. Injection of pseudoginseng and gardenia, from which TLJN is derived, has been shown to activate Aβ-degrading enzymes present in the cortex and hippocampus, primarily insulin degrading enzyme (IDE) and neprilysin (NEP). These studies have suggested that the active ingredients of pseudoginseng and gardenia assist in Aβ clearance [39], which then improves memory. In the APPV7171 transgenic mouse model of AD [40], TLJN was found to enhance learning and memory in the Morris water maze. Previous studies into the mechanisms of TLJN have concluded that the herbal extract can increase neuronal numbers in the hippocampus, raise the expression of synaptophysin in synaptic vesicles, and improve neuronal plasticity [41]. Furthermore, we also found that the level of endogenous formaldehyde is negatively related with human cognition and learning [42]. Together, this suggests a role for TLJN in both treating and preventing AD.

In conclusion, we found that GP, as one of the main components of TLJN, decreases endogenous FA levels *in vitro*. GP can protect N2a cells from the toxic effects of FA through activation of the Akt pathway. While it has so far not been determined whether and to what degree FA contributes to the pathology in AD, our data suggest TLJN and its active ingredient, GP, as promising leads for the detoxification of FA.

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Supporting Information

Table S1 Data from flow cytometry using the AnnexinV-FITC/PI apoptosis detection kit

Table S2 Statistically analytic data for apoptosis with drugs in the presence of formaldehyde

Table S3 RT-qPCR analytic data for Akt

Table S4 RT-qPCR analytic data for FOXO3

Table S5 RT-qPCR analytic data for Bcl2

Table S6 RT-qPCR analytic data for P53

Figure S1 The scheme for the usage of drugs.

Figure S2 Tong Luo Jiu Nao rescues the morphology and increases the number of formaldehyde-treated N2a cells. Conditions were as for Figure 6, except cellular morphology (A) and cell density (B) were GP+FA, TLJN+FA, PNS+FA, FA alone, and saline as indicated (provided by Sun et al. [23]). Cells with no treatment were used as control. Data are expressed as mean±SD from three independent experiments. **, $P<0.01$ vs. the control.

Figure S3 Intact 18S and 28S ribosomal RNA. Conditions for the treatment of cells were as described in Figure 1. Total RNA was extracted from N2a cells using Trizol reagent. The integrity of RNA was examined by identifying intact 18S and 28S ribosomal RNA bands by agarose gel electrophoresis.

Figure S4 Changes in the expression of Akt measured by RT-qPCR analysis. Conditions were the same as in Figure 7 except that Akt was measured.

Figure S5 Changes in the expression of FOXO3 measured by RT-qPCR analysis. Conditions were the same as in Figure 7 except that FOXO3 was measured.

Figure S6 Changes in the expression of Bcl2 measured by RT-qPCR analysis. Conditions were the same as in Figure 7 except that Bcl2 was measured.

Figure S7 Changes in the expression of Bcl2 measured by RT-qPCR analysis. Conditions were the same as in Figure 7 except that p53 was measured.

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